3/21/97

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 71007/137/USGO

In re patent application of
Apurba BHATTACHARJEE et al. Group Art Unit: 1802
Serial No. 08/230,402 Examiner: H. Sidberry
Filed: April 20, 1994
For: VACCINE AGAINST GRAM-NEGATIVE BACTERIAL INFECTIONS

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Alan S. Cross, M.D., declare and say as follows:

1. I am the Alan S. Cross shown as coinventor on the captioned patent application.

2. I have had twenty three years of experience in the field of vaccines directed against bacterial infections. My *curriculum vita* is enclosed.

3. I have read in detail Examiner Paper No. 13, an Office Action in the captioned application mailed October 29, 1996 by Examiner H. Sidberry.

4. In my opinion, there is no basis for the Examiner's assertions that Zollinger *et al.*, United States Patent No. 4,707,543 anticipates or makes obvious the present invention; the differences are striking at every level, as I will outline below:

4.1. In lines 4-5 of the Zollinger Abstract, it is stated that the patent is to products useful as vaccines against infection ". . . by the <u>same</u> bacteria" . . . and protecting

In sharp contrast, our vaccine protects against heterologous infections. See, Example 10, p.20 and Example 11, p.22. This is now a limitati n on all claims.

4.2 Zollinger is concerned with the interaction of vaccine components with the homologous bacteria from which the vaccines are derived.

4.3 Zollinger provides no evidence of cross-protection.

4.4 Zollinger provides no evidence of passive immunization, which is a property of the present vaccine.

4.5 Zollinger recites that the LPS portion of the vaccine can be substituted with the LPS of other Gram-negative bacteria, including *E. coli*. Zollinger implies that these LPSs could provide only type-specific protection. In other words, if the LPS were obtained from *E. coli* 018, then it would be effective only against infection with *E. coli* 018. No data is presented on this subject by Zollinger, and no discussion is provided. It would not be apparent from the Zollinger disclosure that the substitution of the meningococcal polysaccharide or LPS with that of *E. coli* would provide heterologous protection.

5. A second element of the claimed invention that is not anticipated or obviousness-making by Zollinger, is the present role of OMP strictly as an adjuvant. OMP induces no protective activity of its own. Rather, it maintains the LPS in a proper spatial configuration such that relevant cross-reactive epitopes are exposed in a manner different then when they simply are conjugated to protein given alone. Thus, the concepts of the protectiv antibodies are quit different between Zollinger and ourselves.

6. The pres nt type of antibody induced is also different. In the Zollinger patent, what were produced were

I hereby declare that all statem nts made her in of my own knowledge are true and that all statem nts mad on inf rmation and b lief are believed t be tru ; and further that thes statements were made with the knowledge that willful fals statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of th United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

Respectfully submitted,

3/2/97
Date

Alan S. Cross, M.D.

6/19/98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 71007/137/USCO

In re patent application of
Apurba BHATTACHARJEE et al.
Serial No. 08/230,402
Filed: April 20, 1994
For: VACCINE AGAINST GRAM-NEGATIVE BACTERIAL INFECTIONS

Group Art Unit: 1641
Examiner: S. Loring

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Alan S. Cross, M.D., declare and say as follows:

1. I am the Alan S. Cross shown as coinventor on the captioned patent application.

2. I am experienced in the field of vaccines directed against bacterial infections. My curriculum vita is appended to my prior declaration.

3. Zollinger discloses vaccines comprising outer membrane protein and polysaccharide, in which the polysaccharide portion of the vaccine can be capsular polysaccharide or lipopolysaccharide (LPS) of any Gram-negative bacteria, with *E. coli* being just one of the possibilities. By contrast, the present claims recite combinations of outer membrane protein (OMP) derived from *N. meningitidis* and purified, detoxified LPS endotoxin derived from a particular mutant strain of *E. coli* that lacks O-polysaccharide sidechains, the J5 strain.

4. Combinations of OMP derived from *N. meningitidis* and purified, detoxified LPS endotoxin derived from *E. coli* strain J5 provide unexpectedly superior protection against gram-negative sepsis as compared to combinations of OMP with LPS purified, detoxified endotoxins from other strains of *E. coli*.

Serial No.: 08/886,044

In our studies, we have complexed OMP derived from N. meningitidis with other lipopolysaccharides, including LPS endotoxin derived from a strain of Brucella and from E. coli 018 (EC018). Ten mice/group were immunized with PBS or with 20 µg of vaccine (OMP, Brucella-OMP, J5-OMP, or EC018-OMP) at day 0, day 14 and day 28. EIA against all vaccine antigens, lipid A and Re LPS was done on sera drawn prior to challenge. Seven days after the immunization protocol was completed, the mice were challenged with 100 ng of EC018 lipopolysaccharide and 20 mg galactosamine intraperitoneally. Thus, challenge was homologous with respect to mice immunized with EC018-OMP, while challenge was heterologous with respect to mice immunized with J5-OMP.

5. There were no survivors among mice immunized only with OMP. No protection was provided by immunization with Brucella-OMP. Some protection was expected for mice immunized with EC018-OMP, since challenge was with the homologous strain. Sixty percent survival ($p=0.01$) was observed in the group of mice immunized with EC018-OMP. In mice immunized with J5-OMP, however, survival was 90% ($p=0.0001$), i.e., vaccination with J5-OMP provided 50% greater protection than vaccination with EC018-OMP. This was particularly surprising in view of the fact that J5-OMP vaccine was providing protection against infection by a heterologous strain (EC018) whereas EC018-OMP was providing protection against the same strain. LPS endotoxin from E. coli J5 in combination with OMP from *N. meningitidis* clearly provides protection that is markedly superior to LPS endotoxin from other strains of E. coli in combination with OMP from *N. meningitidis*.

6. A clinical protocol to test the safety of the present vaccine for immunising a subject against infection by heterologous Gram-negative bacteria or against lipopolysaccharide (LPS) endotoxin-mediated pathology has been written for Phase I trials in humans, and has been approved by (1) the Walter Reed Army Institute of Research (WRAIR)

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Scientific Review Committee; (2) th WRAIR Institutional Review Board (IRB); and (3) the Surgeon General's Human Subjects Research Review Board (pending only the formality of my being credentialed at WRAIR so that I may act as principal investigator). My co-inventor Dr. Bhattacharjee has consulted with Dr. Richman of the FDA about the specifics of the protocol, who suggested minor modifications to the Phase I trial. These modifications were incorporated, and the protocol will be submitted, along with the IND application, to the FDA.

I further declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of any patent that may issue based on them.

Respectfully submitted,

Alan S. Cross

June 19, 1998
Date

Alan S. Cross, M.D.

1/14/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 71007/137/USGO

In re patent application of

Apurba BHATTACHARJEE *et al.* Group Art Unit: 1641

Serial No.: 08/886,044 Examiner: S. Devi

Filed: June 20, 1997

For: VACCINE AGAINST GRAM-NEGATIVE BACTERIAL INFECTIONS

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I, **ALAN S. CROSS, M.D.**, of 6810 Brookville Road, Chevy Chase, Maryland 20815, do solemnly and sincerely declare that:

1. I am one of the inventors of the inventions disclosed and claimed in the patent application captioned above. My *Curriculum Vitae* is attached to a prior declaration.

2. Attached as Exhibit 1 are Methods and Results relating to active immunization with a detoxified *Escherichia coli* J5 LPS-Group B meningococcal outer membrane protein complex vaccine, and subsequent challenge with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

3. Active immunization with J5 dLPS/OMP vaccine produced a prompt and sustained anti-core glycolipid antibody level that was generally in 100-fold excess of pre-immunization baseline levels. Twenty-four hours after onset of bacteremia, antibody levels decreased, but then rapidly recovered t , and remained at, pre-infection levels. Active immunization with J5 LPS/OMP vaccine induced greater than 800 ELISA units/ml of antibody at the onset of neutropenia, nearly 4 weeks after the last dose of vaccine, and this

level persisted throughout the entire period of neutropenia, for up to 80 days after the initial immunization. This is in distinct contrast to results achieved by passive immunization with antibodies, where initial levels of 800 ELISA units/ml of antibody dropped to less than 200 ELISA units/ml of antibody by 24 hours.

4. Immunization did not prevent either systemic infection or initiation of sepsis, but it did reduce the likelihood of a lethal outcome following infections with both heterologous strains of bacteria. Vaccinated animals challenged with *Pseudomonas aeruginosa* had an overall survival rate of 48% compared to 7% for saline treated control animals. A similar result ensued with *Klebsiella pneumoniae* challenge, with a 64% survival rate for vaccinated animals versus a 13% survival rate for control animals.

5. Vaccinated animals had the same frequency and magnitude of bacteremia from the challenge strain as the control group, but had significantly lower levels of bacteria in liver and spleen than control animals. I hypothesize that antibodies generated in response to the vaccine promote the uptake and killing of bacteria from the blood by tissue.

6. In addition to the decreased bacterial levels in liver and spleen, there was a significantly lower level of circulating endotoxin at the onset of fever in vaccinated animals as compared to control animals. While endotoxin levels increased in both groups at 24 hours, they were still lower than those of the control group. The lower level of circulating endotoxin may be due in part to promotion of LPS clearance from the circulation.

Serial No. 08/886,044

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

1/12/99
Date

Alan S. Cross, M.D.

METHODS

Bacterial Challenge. Two bacterial pathogens were used in these experiments; *Pseudomonas aeruginosa* 12.4.4. and *Klebsiella pneumoniae* K2. *P. aeruginosa* 12.4.4. (originally provided by A. McManus; United States Army Institute of Surgical Research, San Antonio, TX) is a serum-resistant, human blood stream isolate of *P. aeruginosa*. The organism belongs to Fisher-Devlin-Gnabasik immunotype 6. The organism was stored in 10% glycerin at -70°C until ready for use. The day prior to the oral challenge, the isolate was incubated overnight in trypticase soy broth (TSB:Becton Dickinson, Cockeysville, MD) at 37°C . The following day bacteria were suspended in normal saline and adjusted spectrophotometrically to an inoculum size of 10^6 CFU/ml. This dose exceeds the LD_{90} for this experimental model in previous studies (6-8).

Klebsiella pneumoniae K2 (strain B5055) is a serum-resistant, rodent-virulent, encapsulated strain of *K. pneumoniae* (originally obtained from Drs. Frits and Ida Orskov, Staatsserum-Institut, Copenhagen Denmark). The organism was stored and prepared as described above with exception that the challenge dose was 10^7 CFU/ml since preliminary studies demonstrated that a higher inoculum was necessary to achieve a dose that exceeds the LD_{90} in this animal model.

Vaccine. The vaccine used in these experiments is a non-covalent vaccine consisting of detoxified *Escherichia coli* J5 lipopolysaccharide and *Neisseria meningitidis* group B outer membrane protein (7). When this de-O-acylated J5 lipopolysaccharide is complexed with the OMP from group B, *N. meningitidis*, it remains highly immunogenic and is a well-tolerated in experimental animals (7). The vaccine

was stored at 4°C until ready for use. The vaccine was administered at a dose of 20 μg subcutaneously at intervals of 0 and 4 weeks (two dose schedule) or 0, 2 and 4 weeks (3 dose schedule). The temperature was checked by infra-red thermometry (Horiba) 24 hr prior, 1, 2, 24 and 48 hr after each immunization. The weights were checked weekly.

Animal Model. The basic design of the neutropenic rat model has been described in detail previously (6-8). Briefly, the female, non-pregnant, specific pathogen-free, albino, Sprague-Dawley rats weighing between 125 and 150 gm (Charles River Breeding Labs, Wilmington, MA) were maintained in filtered, biological safety cages and allowed to eat and drink ad libitum. After a seven day control period, the animals underwent baseline blood sampling and then were immunized the J5 dLPS/OMP complex without the addition of an adjuvant. Two weeks after the last dose of the vaccine, repeat blood sampling was performed to determine vaccine responsiveness. Four weeks after the last dose of vaccine, animals were then rendered neutropenic with cyclophosphamide (Bristol-Meyers, Evansville, IN) at a dose of 100 mg/kg (time 0) IP followed by a second dose of 50 mg/kg IP 72 hours later to induce neutropenia.

Cefamandole (100 mg/kg) (Eli Lilly, Indianapolis, IN) was given IM beginning 96 hours before the first dose of cyclophosphamide to facilitate colonization of the alimentary tract with the challenge strain of *P. aeruginosa*. Ampicillin (Sigma, St. Louis, MO) was given at a dose of 25 mg/kg IM and orally on an every other day basis to disrupt colonization resistance against *Klebsiella pneumoniae*.

The activity of the vaccine was also tested in the presence of active antimicrobial therapy against the challenge strain of *P. aeruginosa*. At the onset of fever ceftazidime (50 mg/kg) (Glaxo W llcome, Research Triangle Park, NC) was given IV in one

experimental group with (n=18) and without (n=10) the active vaccine. The treatment was given intravenously at a low dose (to promote antibiotic-induced endotoxin release [9]) every 12 hours for 48 hours after the onset of fever in these septic animals.

The bacterial challenge was given orally via orogastric tube prepared from polyethylene tubing (Intramedic PE, 160: Clay Adams Division, Becton Dickenson, Parsippany, NJ). The bacterial challenge was given on day 0 (the first dose of cyclophosphamide) and again on days 2 and 4. Phosphate buffered saline (PBS) was given as a control for the intravenous injections and for the vaccine placebo groups. A bacterial suspension was prepared to deliver 1 ml of 10^6 CFU *P. aeruginosa* 12.4.4 or 10^7 CFU *K. pneumoniae* K2 for each experimental group.

All manipulations were done under light CO_2 anesthesia to minimize any stress or trauma to the animals. Before onset of neutropenia, a patch of fur approximately 4x4 cm was shaved off the lateral thoracic region of the animal to allow for accurate and repeated body temperature recordings. A Horiba non-contact digital infrared thermometer (Markson Science - Phoenix, AZ) was used to monitor the animal's body temperature several times daily. Fever generally occurred in infected animals 4-5 days after the initial dose of cyclophosphamide; fever was defined as a body temperature measurement >38.0°C. The experiments were approved by the Brown University Animal Care Committee and were in accord with national guidelines for laboratory animal facilities and care.

Blood determinations and necropsy studies. Blood samples were obtained from the retro-orbital plexus of each animal under CO_2 anesthesia prior to immunization; two weeks after the four week immunization schedules; two days prior to the first dose of

cyclophosphamide; at the onset of fever; and 24 hours after the onset of fever. Each blood sample was tested for quantitative bacterial counts, serum endotoxin levels, and anti-J5 antibody levels. Quantitative bacteriology was performed using standard methods with serial dilutions of whole blood performed in TSB. The limit of detection was 10 CFU/ml of blood. Blood and tissue specimens from animals challenged with *P. aeruginosa* 12.4.4 were plated on Pseudomonas Isolation agar (Difco, Detroit, MI). Non-lactose-fermenting, oxidase-positive colonies were identified and immunotyped with polyvalent *P. aeruginosa* antisera (Difco, Detroit MI). In *K. pneumoniae* K2 challenge experiments, cultures were plated on Simmon's Citrate media (Becton Dickenson, Cockeysville, MD) and then characterized using standard microbiologic methods. The bacterial colony counts from the liver and spleen were measured separately for each animal but since the colony counts from the two sites were so similar, the results were combined and reported compositely as CFU/gm tissue.

Endotoxin levels were measured in serum samples which were heat-treated to 70°C after a 1:10 dilution in endotoxin-free water. Endotoxin measurements were determined by turbidimetric quantitative limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). Anti-J5 antibody titers were measured using an ELISA method previously described (6,7).

Each animal was examined daily throughout the experiment until 14 days after cyclophosphamide treatment. Previous experiments (6-8) have shown that the period of neutropenia (<50 granulocytes/mm^3) induced by this dose regimen of cyclophosphamide begins three days after the first dose of cyclophosphamide and extended until days 10-12. Animals that remain alive for >14 days after the cyclophosphamide treatment were

considered long-term survivors. All animals that succumbed during the course of the experiment were subjected to necropsy examination with quantitative cultures obtained from the cecum, liver, spleen, and lung tissue. Animals that survived the experiment were sacrificed and a necropsy examination with quantitative cultures of the same organ samples are obtained.

EndoCab assay. This assay for antibody to core glycolipids was performed as previously described (10,11). Briefly, 96 well microtiter plates were coated with a mixture of one rough LPS (incomplete outer core of chemotypes Rc or Rb) from *E. coli*, *Salmonella*, *Klebsiella* and *Pseudomonas aeruginosa*. Serum samples were added to the wells and anti-core glycolipid antibodies bound were detected by alkaline-phosphatase conjugated goat anti-rabbit IgG.

Data Analysis and Statistical Methods. Survival functions were measured using Kaplan-Meier plots and differences in survival time were measured using a non-parametric Kruskal-Wallis one-way analysis of variance. Numeric data was compared using the Mann-Whitney U-test. Numeric data are expressed as mean ± standard error; p values <0.05 was considered significant.

RESULTS

Vaccine Response. Both 2 dose and 3 dose vaccine schedules were studied in preliminary experiments. Animals vaccinated with the J5 dLPS/OMP complex vaccine (n=40) experienced no febrile reactions for up to 48 hrs after each immunization and had feeding and weekly weight gain patterns which were not different from the saline-

immunized control groups (n=31). The two vaccine schedules resulted in anti- J5 antibody lev ls which exceeded the target antibody response of 800 ELISA units (Figure 1). This level of antibody response was predicted to be protective based upon previous experiments with passively administered, rabbit-derived antisera (6). Since the 3 dose vaccine regimen resulted in significantly greater ($p<0.05$) antibody titers (2,440±526 ELISA units, n=40) than the 2 dose regimen (840±175 units, n=15), the 3 dose schedule was exclusively used in subsequent challenge experiments with *P. aeruginosa* and *K. pneumoniae*.

Vaccine effects on survival. Pseudomonas infection in the absence of ceftazidime. The circulating granulocyte levels were below 50 granulocytes/mm^3 in a sample of animals (n=10) tested 3 days after treatment with cyclophosphamide. Antibody elicited by this vaccine protected neutropenic rats from lethal *Pseudomonas* infection when passively infused as treatment at the onset of fever (7). We therefore examined whether this vaccine induced protection against lethal sepsis when actively administered as prophylaxis before the induction of neutropenia and infection. A Kaplan-Meier survival plot of vaccinated and control groups of neutropenic animals who received *Pseudomonas aeruginosa* 12.4.4. oral challenge is depicted in Figure 2. Vaccinated animals had an overall survival rate of 48% (13/28) while saline treated control animals had a survival rate of 7% (2/29) ($p<0.01$).

After the third dose of vaccine, there was a prompt (by day 35, 7 days following the last dose of vaccine) and sustained (>12 weeks) anti-core glycolipid antibody levels which were generally 100-fold in excess of pre-vaccine baseline levels (Table 1, see

below). Antibody titers diminished slightly over the course of bacteremic infection in *Pseudomonas aeruginosa*- challenged animals (Table 1). Twenty-four hr after infection anti-J5 LPS antibody levels decreased, but then rapidly recovered to pre-infection levels and remained elevated throughout the duration of the experiment (3 months). The saline-treated control animals had anti-J5 antibody levels which were at the limits of detection throughout the experimental period (3 months).

Circulating levels of bacterial endotoxin were undetectable or very low prior to the onset of infection in vaccinated and control animals challenged with *P. aeruginosa* in the absence of ceftazidime therapy (Figure 2). Vaccinated animals had a significantly lower level of endotoxin at the onset of fever during the course of *P. aeruginosa* infection in these immunocompromised animals. However, endotoxin levels were elevated to a similar degree in vaccinated and control groups after 24 hours of continued fever and overt illness in these neutropenic animals (Figure 2).

Pseudomonas infection in the presence of ceftazidime. Since antibiotic treatment may liberate endotoxin from the dying bacteria (9), we tested the ability of actively-induced antibody to protect animals from lethal sepsis under conditions in which there may be an acute endotoxin load. A similar level of protection was observed in animals who received vaccine and ceftazidime at the onset of fever (Figure 4) as was observed in animals receiving vaccine alone (Figure 3) (i.e. approximately 60 % survival). Ceftazidime was highly active *in vitro* against this strain of *P. aeruginosa* 12.4.4 (MIC=0.25 μg/ml). Ceftazidime-treated animals cleared the Pseudomonas bacteremia (0 cases of bacteremia/10 animals) after 24 hr of therapy, yet this dose of ceftazidime, while prolonging survival compared to animals not receiving antibiotics, was unable ultimately

to protect these neutropenic animals from lethality (Figure 4). In contrast, the J5 dLPS/OMP vaccine significantly improved mortality (11/18 survived, $p<0.01$) in combination with ceftazidime (Figure 4).

At the onset of fever and 24 hr later, endotoxin levels remained significantly elevated in non-vaccinated animals treated with ceftazidime (n=10; 5.45±2.2 ng/ml) and these circulating endotoxin levels were not significantly different from the saline-immunized control group (n=4; 7.7±3.3 ng/ml) (p=NS). Ceftazidime-treated animals who also received the J5 dLPS/OMP vaccine, however, had the lowest endotoxin levels within the first 24 hr after fever onset (n=18; 2.9±1.5 ng/ml) ($p<0.05$, compared to non-immunized animals).

Klebsiella infection in the absence of ceftazdime. Previous studies in this neutropenic rat model used *Pseudomonas aeruginosa* as the primary challenge strain. If this J5 dLPS/OMP vaccine is to have broad clinical applicability in the prevention and/or treatment of gram-negative bacterial sepsis, it should be efficacious against infections caused by other heterologous gram-negative bacilli. *K. pneumoniae* challenge was highly lethal in the saline control group (Figure 5). The Kaplan-Meier survival plots of animals that received the J5 dLPS/OMP complex vaccine (n=14) and the control group (n=15) is depicted in Figure 5. The vaccine provided a highly significant survival protection in these neutropenic animals (9/14 of immunized animals survived v. 2/15 of saline-immunized) ($p<0.005$). As was observed in animals infected with Pseudomonas, there was a decrease in anti-J5 LPS antibody levels at 24 hr after onset of fever in Klebsiella-infected animals, but here, too, the levels returned to pre-febrile levels (data not shown).

Thus active immunization with the J5 dLPS/OMP vaccine pr vided a survival advantage for infection with both *Pseudomonas* and *Klebsiella* species.

Endotoxin levels in the circulation of animals infected with *Klebsiella pneumoniae* K2 were significantly reduced in the vaccine-treated group. Blood levels of endotoxin 24 hr after the onset of fever were 0.75±.43 ng/ml in the vaccinated animals while the endotoxin levels were 4.9±1.5 ng/ml in the control group ($p<0.01$).

Bacterial load. Multisystem infection with either *P. aeruginosa* 12.4.4 or *K. pneumoniae* K2 occurred invariably in the control group, resulting in a >90% mortality (Figures 3-5). In each experiment, vaccinated animals had the same frequency and magnitude of bacteremia from the challenge strain as the control group. When measured within the first 24 hr after fever onset, the quantitative level of bacteremia in *P. aeruginosa*-challenged animals was 76±23 CFU/ml (vaccine group) and 205±150 CFU/ml (control group) (p=NS). The quantitative level of bacteremia following *K. pneumoniae* challenge was 583±280 CFU/ml (vaccine group) vs. 412±201 CFU/ml (control group) (p=NS).

Despite no significant differences in circulating levels of bacteremia, quantitative bacterial counts of organ tissue cultures revealed that vaccinated animals had significantly lower tissue levels of the challenge organism when compared to the control groups in both the *Pseudomonas* and *Klebsiella*-challenged animals. In the animals challenged with *P. aeruginosa* in the absence of ceftazidime, the tissue levels in the vaccine group (n=28) were 401±177 CFU/mg tissue, while the control group (n=29) had tissue levels of 2,342±693 CFU/mg ($p<0.01$). In ceftazidime-treated animals challenged

with *P. aeruginosa* the organ bacterial colony c unt was 571±352 CFU/mg f r those receiving both vaccine and antibiotic (n=18), 2,789 ±1,726 CFU/mg in those receiving antibiotic alone (n=10) and 2,665 ± 1,994 CFU/mg in animals receiving neither vaccine nor antibiotic (n=4) ($p<0.01$). The quantitative tissue levels in *K. pneumoniae* K2-challenged animals in the vaccine group (n=14) was 127±101 CFU/mg while the levels in the control group (n=15) was 3,683±224 CFU/mg ($p<0.001$).

EndoCab assay. In separate experiments the sera from rabbits immunized with two doses of this J5 dLPS/OMP vaccine was tested for its ability to bind to a mixture of Rc or Rb chemotype LPS (10,11). Unlike normal rabbit IgG, the vaccine-immune sera had easily detectable titers of antibody to the LPS mixture, as did the sera from rabbits immunized with a mixture of core LPS antigens. Thus, the J5 dLPS/OMP vaccine induced a population of antibodies that bound to a similar panel of antigens to which serum from an unrelated vaccine composed of core LPS antigens bound.

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Legends

Figure 1. **Antibody levels to J5 LPS following 2- and 3-dose immunization regimens.** Rats were immunized with the J5 dLPS/OMP vaccine given subcutaneously (20 μg/dose) at time 0 and 4 weeks (2 dose) and at time 0, and at 2 and 4 weeks (3-dose). Control rats were immunized with saline. Serum was harvested at 6 weeks and antibody levels determined by ELISA (see Methods). While both immunization regimens induced antibody above a previously determined protective level (2), the antibody level induced by the 3 dose regimen was significantly greater that that induced following 2 doses ($p<0.0.05$).

Figure 2. **Circulating serum endotoxin levels in J5 dLPS/OMP vaccine- and saline-immunized rats.** Rats were immunized at time 0, and at 2 and 4 weeks. At 14 days following the last vaccine dose, animals were given the first dose of cyclophosphamide and of *Pseudomonas*. Animals were then followed every 12 hours for the onset of fever. Serum drawn at the onset of fever (typically days 5-6 after the first dose of cyclophosphamide) and at 24 hr later, were evaluated for endotoxin levels by a *Limulus* amebocyte assay.

Figure 3. **Effect of J5 dLPS immunization on survival following challenge with *Pseudomonas*.** Animals were immunized with either saline or J5 dLPS/OMP vaccine at time 0, and at 2 and 4 weeks. Fourteen days after the last immunization, animals were given the first doses f cyclophosphamide and of *Pseudomonas*, and followed for the

onset of fever. A Kaplan-Meier survival curve was plotted. Two of 29 animals in the control group survived, wher as (13/28) of immunized rats survived ($p<0.01$) t the conclusion of the experiment, when the neutropenia resolved.

Figure 4. **Effect of J5 dLPS immunization on survival of rats infected with *Pseudomonas* and given 4 doses of ceftazidime every 12 hr at the onset of fever.** Rats were immunized and treated with cyclophosphamide and given bacteria as described in Figure 3. At the onset of fever, however, ceftazidime, which binds to penicillin binding protein 3, was given to induce the release of endotoxin from the bacteria. Rats that received neither vaccine nor antibiotic all died by days 6 and 10 respectively. There was an increase in survival (11/18) among animals that were immunized with J5 dLPS/OMP vaccine and given ceftazidime .

Figure 5. **Increased survival among rats immunized with J5 dLPS/OMP vaccine and challenged with *Klebsiella*.** Rats were immunized with 3 doses of vaccine as described earlier (Figures 2 and 3). Fourteen days after the last dose of vaccine animals were given the first dose of cyclophosphamide and of *Klebsiella* by gavage. Instead of cefamandole, animals were treated with ampicillin to overcome colonization resistance. Fever and survival was followed for 12 days. Increased survival was observed among rats actively immunized with the J5 dLPS vaccine.

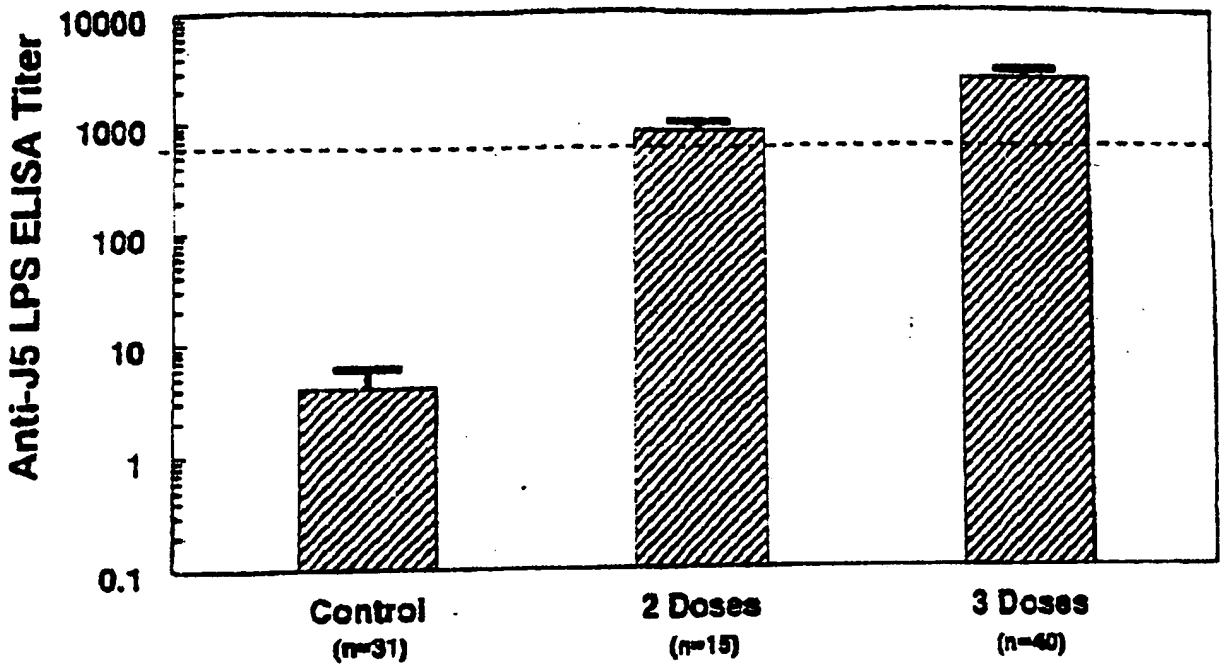
Figure 6. **Immunization with J5 dLPS elicited antibodies that reacted with a mixture of core glycolipid antigens from heterologous gram-negative bacteria.**

Antibody levels to a mixture of 4 different Rc or Rb LPS (one each from *E. coli*, *Salmonella*, *Pseudomonas aeruginosa* and *Klebsiella*) each complexed to polymixin B. The sera from rabbits (130, 131, 134) immunized with a mixture of core antigens at monthly intervals x6 before being bled were compared to sera from two rabbits (anti-J5 dLPS, lots #1 and 2) immunized at time 0 and day 28 with the J5 dLPS/OMP vaccine. The level of antibody to core LPS structures in normal rabbit IgG (Sigma, St. Louis, MO) is shown for comparison.

TABLE 1. E. COLI J5 ANTIBODY LEVELS (ELISA UNITS)

TREATMENT	PRE-VACCINE (day 0)	POST-VACCINE (day 35)	ONSET OF SEPSIS	24 HR. POST ONSET OF SEPSIS	RECOVERY* (14 days after sepsis)
Vaccine (n=28)	6.4 ± 2.0	2852 ± 191**	2467 ± 527**	1827 ± 488**	2690 ± 663**
Control (n=29)***	7.5 ± 1.9	5.7 ± 3.0	8.5 + 2.8	7.6 ± 2.8	7.9 ± 3.1

Animals were immunized subcutaneously at days 0, 14 and 28 with 20 µg of the J5 dLPS/OMP vaccine or with normal saline. IgG antibody levels to J5 LPS were measured 7 days after the third dose. Thirty-three days after the third dose (61 days after the first dose of vaccine) animals were given the first dose of cyclophosphamide and *Pseudomonas aeruginosa* (see Methods). Additional antibody levels were measured at onset of sepsis (day 66), 24 hours later (day 67) and in surviving animals, at recovery (day 80). *Data from the 2 long term survivors from control group and from 13 long term survivors in the vaccine group; **$p<0.0001$ compared to pre-vaccine levels; ***p=ns all post-vaccine time points compared to pre-vaccine levels.



J5 dLPS/OMP given SQ (20ug/dose) 0,4wks-2 dose; 0,2,4wks-3 dose

Figure 1

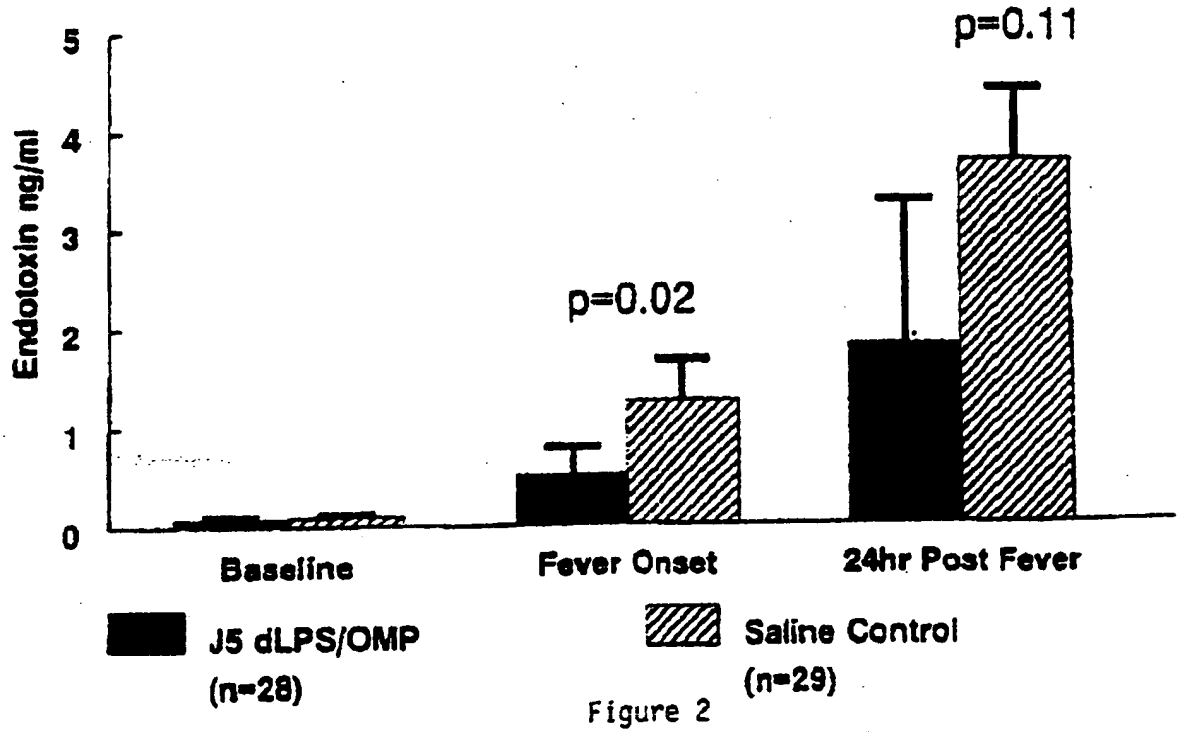


Figure 2

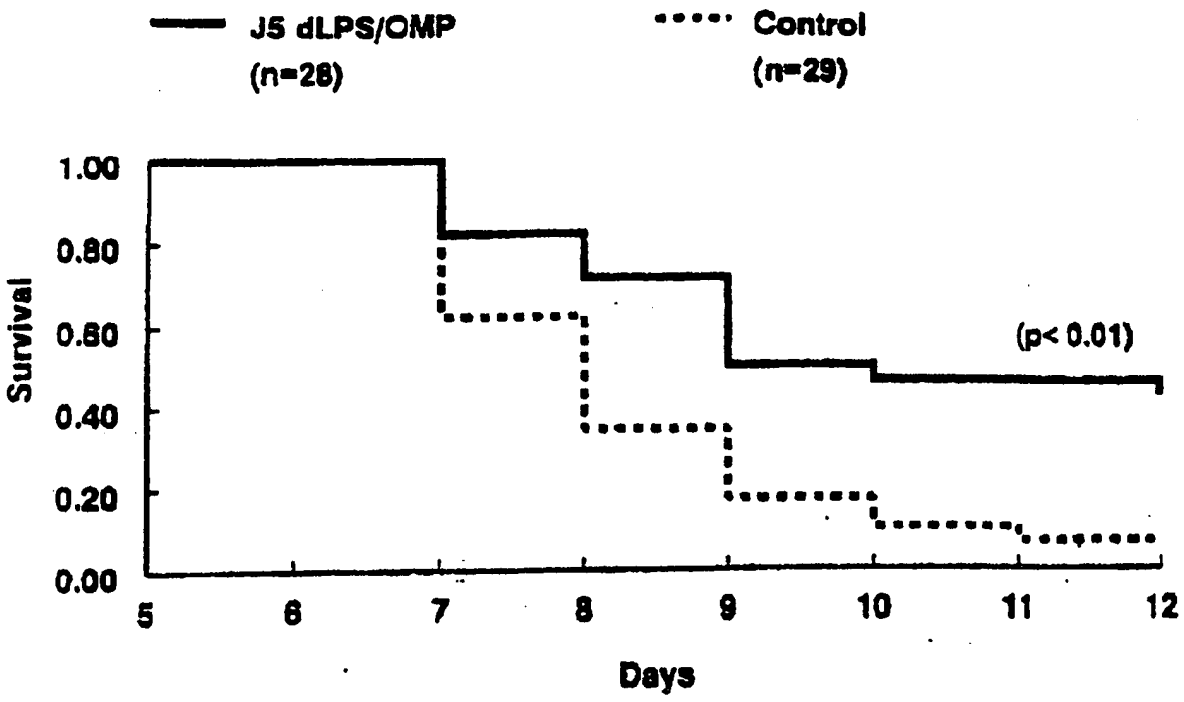


Figure 3

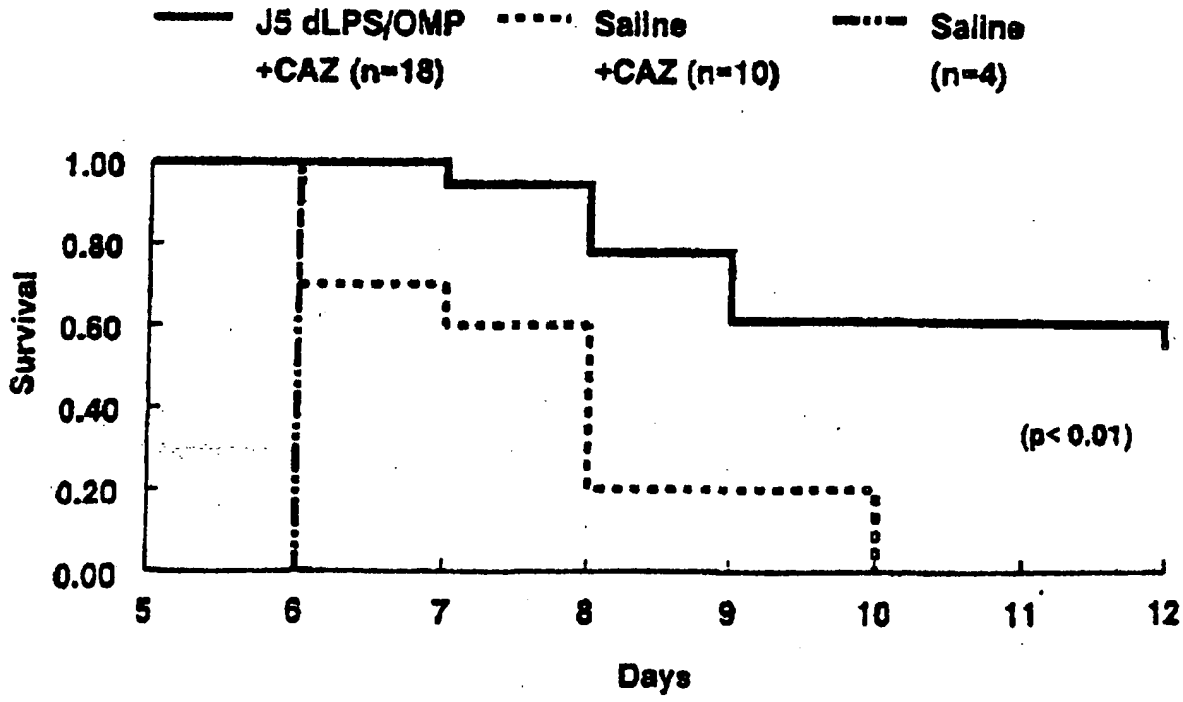


Figure 4

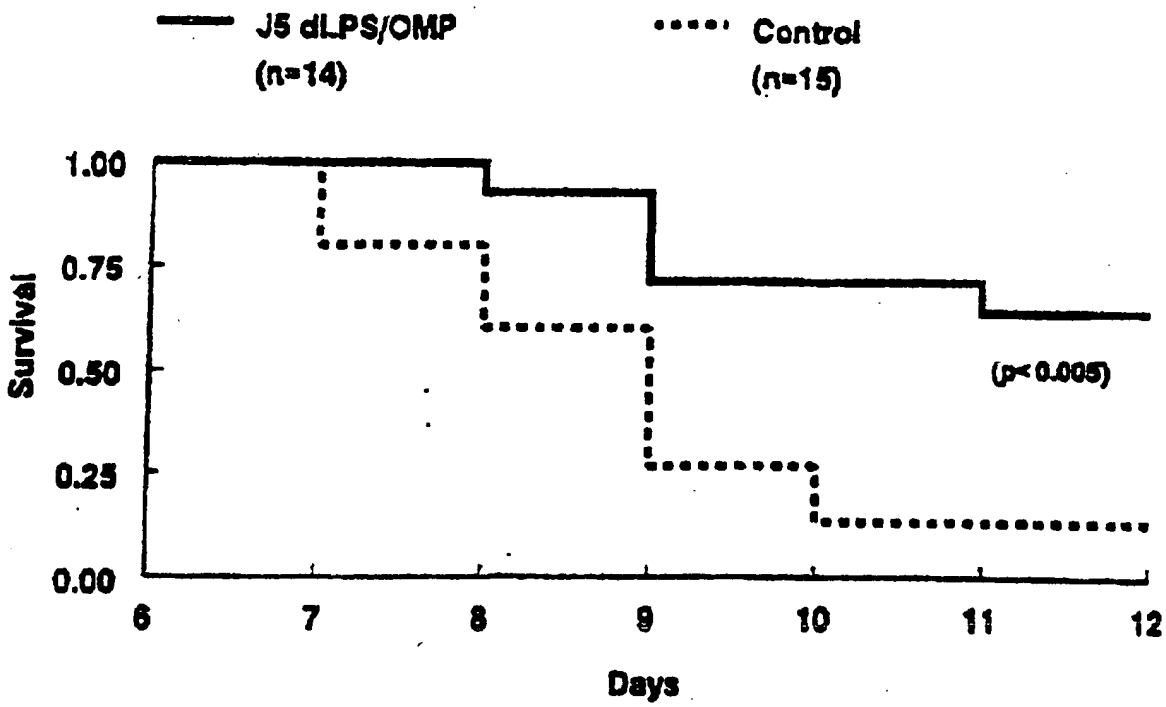

J5 dLPS/OMP
(n=14)
Control
(n=15)
1.00
0.75
0.50
0.25
0.00
Survival
(p< 0.005)
6
7
8
9
10
11
12
Days

Figure 5